



HER2 transmembrane domain mutation: comprehensive characteristics and real-world evidence of treatment response in Chinese lung adenocarcinoma

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Background: *HER2* transmembrane domain (TMD) mutation has been reported as a rare driver mutation associated with advanced stage disease and a poor prognosis in patients with lung adenocarcinoma (LUAD). We aimed to comprehensively profile the genetic landscape and treatment response information of *HER2* TMD-mutant LUAD.

Methods: An in-house database of 7,812 LUAD patients was screened for mutation prevalence. A multi-center cohort of 16 *HER2* V659E-mutant patients and an external cohort of 38 *HER2*-mutant patients from cBioPortal with overall survival (OS) data were analyzed. Eight patients from the in-house cohort were included in the real-world study of treatment response. Molecular docking simulation and binding affinity prediction were performed.

Results: In Chinese LUAD, the prevalence of *HER2* TMD mutation was 0.18% (14/7,812), and 0.14% (11/7,812) for the *HER2* V659E mutation. The most recurrent co-alteration was *TP53* mutation (n=4, 25%) and *HER2* amplification (n=2, 12.5%). TMD-mutant patients were diagnosed at more advance stages (P<0.001) and had poorer OS (median OS 10.0 vs. 61.6 months, HR =7.9, 95% CI: 1.0–61.0, P<0.001) than non-TMD mutations. The overall response rate of targeted therapy, chemo-based therapy, and immunotherapy was 57.1%, 22.2%, and 0%, respectively. We postulated to challenge the resistance

of tyrosine kinase inhibitor (TKI) with another with stronger binding energy to *HER2* and supported the conclusion with a successful case. Additionally, we demonstrated a three-month response to the off-label use of pyrotinib in fifth-line therapy.

Conclusions: Compared with non-TMD mutations, *HER2* TMD mutation is a rare driver mutation with poorer prognosis in LUAD. Targeted therapy is the dominant choice for patients harboring this targetable mutation and longer OS could possibly be achieved through rechallenge with TKI of stronger binding affinity. Response to fifth-line pyrotinib was observed.

Keywords: *HER2* mutation; lung adenocarcinoma (LUAD); prognosis; treatment response; pyrotinib

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Introduction

The comprehensive management of non-small cell lung cancer (NSCLC) has been revolutionized, switching from a one-treatment-fits-all approach to personalized medicine (1). With the advancements in next-generation sequencing (NGS), numerous driver mutations have been revealed and treatments have attained higher precision (2). Tumor NGS reports are detailed into specific alterations and mutation loci, as mutations at different loci of the same gene might lead to different treatment responses (3). Mutations at rare loci acting as driver mutations have also been found.

Erb-b2 receptor tyrosine kinase 2 (*HER2/ERBB2*) is one of the driver genes identified that are susceptible to targeted treatments (4). *HER2* alteration is usually seen as amplification (2–23%) and/or overexpression (11–32%) in lung adenocarcinoma (LUAD), but rarely as mutations (1.6–4%) (5–7). *HER2* mutation is a distinct therapeutic target and a poor prognosis predictor (8–10). Most of the *HER2*-mutant NSCLCs are adenocarcinomas and occur in female never-smokers (8,11,12). Overall survival of *HER2*-mutant NSCLCs is 28.4 months (8) and ranges from 19.2 to 22.8 months among those diagnosed at stage IV (11,13). The gene product of *HER2* is a membrane protein by the same name, which is a receptor tyrosine kinase that forms homodimers or heterodimers to activate downstream pathways (14) and has a transmembrane domain (TMD) where intramolecular interactions occur and lead to dimerization and activation (14). In LUAD, most *HER2* mutations occur in the kinase domain (KD, 2–4%), but rarely in the TMD (0.07–0.20%) (8,15–19). Moreover, activating TMD mutations most frequently affect residues V659 and G660 in exon 17 (20–23).

Most *HER2*-targeting agents such as trastuzumab did not result in comparable efficacy in NSCLC harboring *HER2*

exon 20 mutations as they did in *HER2*-positive breast cancer. A mechanistic explanation is yet to be established but may implicate intratumoral heterogeneity and lower *HER2* expression in *HER2*-mutant NSCLC compared with in *HER2*-amplified breast cancer (24). Therapies for *HER2*-mutant NSCLC is under active development, among which the most promising include reversible or irreversible anti-*HER2* TKIs and antibody-drug conjugates (ADCs) that target *HER2* alone or along with EGFR (25). Patients harboring a *HER2* TMD mutations were reported to respond to *HER2* tyrosine kinase inhibitor (TKI), lapatinib, and afatinib in case reports (15,26); and to the antibody-drug conjugate, ado-trastuzumab emtansine (TDM-1) in a basket trial (9). Due to their low prevalence, it remains to be determined whether TMD mutations would result in a comparable response to targeted therapy as *HER2* KD mutations.

Herein, we demonstrated the comprehensive characteristics and prognosis of the *HER2* TMD-mutant population and illustrated the real-world evidence (RWE) of treatment response from the largest reported LUAD cohort that harbors *HER2* V659E mutation. To enrich the limited clinical data on how to overcome resistance to targeted therapy, we postulated to challenge the resistance of former-line TKI with TKI that has stronger binding energy to the ATP-pocket of *HER2* KD mutants. Additionally, we demonstrated a three-month response to the off-label use of pyrotinib in fifth-line therapy. We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/tlcr-21-107>).

Methods

Database, sample collection, and NGS

We screened the genomic profiles of samples from

7,812 Chinese LUAD patients in the Burning Rock LAVA Open-access Database, profiled between Jan 2014 and July 2019. As an external cohort, a total of 4,587 samples of 4,185 patients was selected from The Cancer Genome Atlas (TCGA) and other studies (27-37) through the open platform cBioPortal for Cancer Genomics (38,39). After deduplication, a total of 2,966 patients with 3,480 samples were included. In brief, 38 *HER2*-mutant patients with overall survival (OS) were deduplicated and included in the final analysis. Protocols of sample collection, DNA extraction, and plasma cell-free DNA preparation were as previously described (40). Captured-based targeted NGS using various panels, including panels with eight, 168, or 520 cancer-related genes (Burning Rock Biotech, Guangzhou, China) were performed according to protocols as previously described (41). The panel gene lists are shown in Tables S1-S3. TMD is defined as 27 amino acids (Ala648 to Leu674), and KD is defined as 274 amino acids (Ile714 to Val987) (42).

Clinical characteristics and response assessment

Patient characteristics, treatments, and outcomes were obtained from the LAVA database with the permission of patients and their physicians in-charge. Patients were followed from the date of diagnosis until death or the last available follow-up. Response to therapy was measured using RECIST v1.1 criteria and OS was calculated from the date of the initiation of treatment until the day of last follow-up or death. All procedures performed in studies involving human participants were in accordance with the Helsinki Declaration (as revised in 2013). The study was conducted under the approval of the Ethics Committee of Peking Union Medical College Hospital (ZS-1329). Written informed consent was obtained from each patient.

Computational methods of simulation and energy analysis of lapatinib, afatinib, and pyrotinib in HER2 KD binding

AutoDock 4.2 (43) was used for molecular docking simulations of lapatinib, afatinib, and pyrotinib and predicting binding affinity with the HER2 KD (PDB code:3RCD). The Lamarckian genetic algorithm (LGA) (44) was used to optimize the binding conformations of compounds. The structure of the lowest predicted binding free energy in the most popular cluster of each compound was selected as the initial conformation for the following molecular dynamic (MD) simulation. MD simulations were implemented by AMBER18 (45). The potentials for

protein and ligands in each complex were generated based on AMBER03 (parm03) forcefield (46), and general amber forcefield (gaff) (47), respectively. Partial atomic charges were assigned using the AM1-BCC charge method (48,49) within the ANTECHAMBER in AMBERTools12 (50). The compound was neutralized with the counterions of Cl⁻ and the whole system was immersed in a truncated octahedron's box of TIP3PBOX water (51). The periodic boundary is 12 Å from any solute atoms. The binding free energy was evaluated by the MMGBSA method, which is based on the following equation (52):

$$\Delta G_{\text{bind}} = G_{\text{complex}} - G_{\text{protein}} - G_{\text{ligand}} = \Delta E_{\text{MM}} + \Delta G_{\text{GB}} + \Delta G_{\text{SA}} - T\Delta S = \Delta E_{\text{vdw}} + \Delta E_{\text{ele}} + \Delta G_{\text{GB}} + \Delta G_{\text{SA}} - T\Delta S \quad [1]$$

where ΔE_{MM} is the molecular mechanics interaction energy between the protein and the ligand, which is comprised of two parts: the electrostatic (ΔE_{ele}) and the van der Waals energies (ΔE_{vdw}). Further, ΔG_{GB} and ΔG_{SA} are the polar and nonpolar contributions of the desolvation free energy upon the construction of the protein-substrate complex. The 200 snapshots taken from the last 6.0 ns MD simulation trajectories of the complex were used to calculate the protein-substrate binding free energy, which was accomplished by the MMPBSA.py program in AmberTools18 (53).

Statistical analysis

Clinical characteristics between the group of *HER2* TMD-mutant patients with OS data and the group of *HER2* non-TMD mutation with OS data were performed with Student *t*-test if the characteristic was a continuous variable; with Chi-square test if the characteristic was a binary variable; and with non-parametric Mann-Whitney test if the characteristic was a ranked variable. Kaplan Meier curves for OS of the two above-mentioned groups were plotted. Pairwise comparisons using log-rank test were performed and the P value was adjusted using the Benjamini-Hochberg method. Cox regression model was used in the univariate and multivariate analysis. All the above-mentioned statistical analyses were conducted in R Studio using Package survminer (v0.4.7; <http://cran.r-project.org/web/packages/survminer/index.html>), ggplot2 (54), and survival (55).

Results

Comprehensive characteristics of the HER2 TMD mutated LUAD

We analyzed the NGS data of 7,812 LUAD patients and

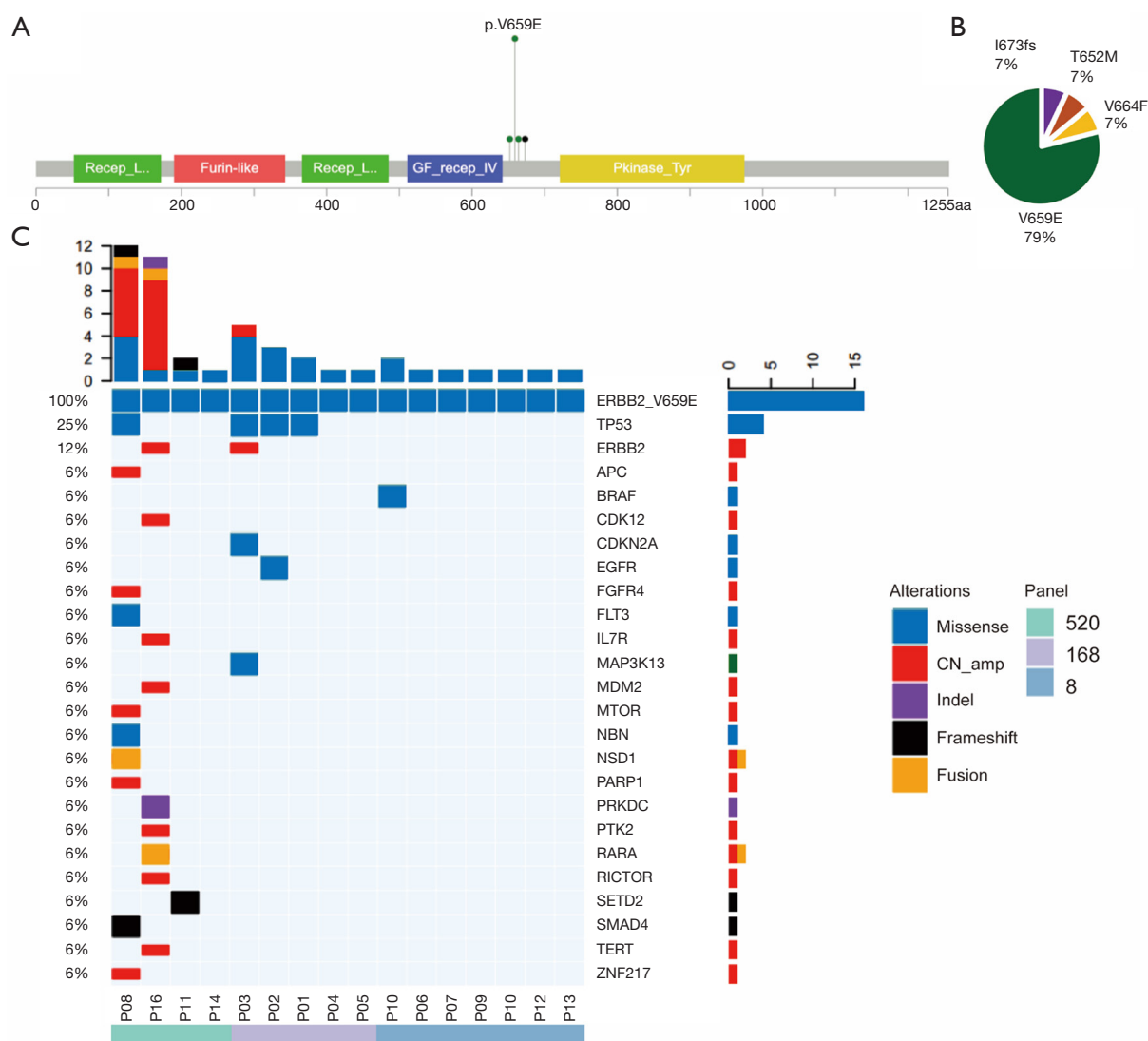


Figure 1 Molecular characteristics of *HER2* TMD mutations. (A) An overview of the *HER2* TMD mutation region in the LAVA database. The mutation region is referred to the Pfam database. (B) Constitution of *HER2* TMD mutation cohort in the LAVA database. (C) Oncoprint of concurrent mutations of oncogenes and tumor suppressor genes in patients with *HER2* TMD mutations. Four patients used 520 cancer-related gene panel, five used 168 cancer-related gene panel, and seven used 8 cancer-related gene panel. Right Y axis shows gene names of the concurrent gene mutation and left Y axis shows the percentage of patients harboring such concurrent mutation. TMD, transmembrane domain.

identified 14 (0.18%) with a *HER2* TMD mutation. These TMD mutations covered four amino acids among the 27 amino acids of the *HER2* TM domain (Figure 1A,B). The most mutations occurred at codon 659, which were mostly V659E mutation (11/7,812, 0.14%). Seven patients from the external cohort of 2,966 NSCLC patients harbored a *HER2* TMD mutation (0.24%) and three of these harbored a *HER2* V659E mutation (0.10%). A retrospective search in medical records from cooperating medical centers assembled

a cohort of 16 *HER2* V659E-mutant patients (Table 1), and detailed characteristics of the patients are shown in Table S4. Five of these patients were not included in the formal analysis, and eight did not have follow-up information. In this cohort, the tumors of all patients were LUAD and harbored a dinucleotide missense mutation, including 10 TT→AA and 6 TT→AG (c.1976_1977). There were equal numbers of males and females (n=8 for each), and 68.75% were non-smokers (n=11). Most of the tumors

Table 1 Clinicopathological and genetic characteristics of cohorts harboring *HER2* TMD or non-TMD mutations

Variable	<i>HER2</i> TMD mutation ¹ (n=16)	<i>HER2</i> TMD mutation with OS data ² (n=10)	<i>HER2</i> non-TMD mutation with OS data ³ (n=36)	P value
Age, mean (SD)	62.75 (10.50)	64.40 (8.58)	64.00 (9.98)	0.93
Sex (%)				0.10
Female	8 (50.00)	2 (20.00)	20 (66.67)	
Male	8 (50.00)	8 (80.00)	16 (53.33)	
Stage (%)				<0.001
I	3 (18.75)	0 (0.00)	24 (80.00)	
II	0 (0.00)	0 (0.00)	6 (20.00)	
III	0 (0.00)	0 (0.00)	5 (16.67)	
IV	13 (81.25)	10 (100.00)	1 (3.33)	
Pathology (%)				0.24
LUAD	16 (100.00)	10 (100.00)	27 (90.00)	
LUSC	0 (0.00)	0 (0.00)	9 (30.00)	
Smoker (%)				0.13
Yes	11 (68.75)	3 (30.00)	25 (83.33)	
No	5 (31.25)	7 (70.00)	7 (23.33)	
Unknown	0 (0.00)	0 (0.00)	4 (13.33)	
<i>HER2</i> alteration				<0.001
TMD	16 (100.00)	10 (100.00)	0 (0.00)	
V659E mutation				
c.1976_1977delTTinsAA	1 (6.25)	1 (10.00)	–	
c.1976_1977delinsAG	6 (37.50)	1 (10.00)	–	
c.1976_1977inv	3 (18.75)	2 (20.00)	–	
c.1976_1978inv	3 (18.75)	1 (10.00)	–	
c.1976_1979inv	2 (12.50)	2 (20.00)	–	
c.1976_1980inv	1 (6.25)	1 (10.00)	–	
non-V659E mutation	0 (0.00)	2 (20.00)	–	
KD	0 (0.00)	0 (0.00)	14 (46.67)	
Others	0 (0.00)	0 (0.00)	22 (73.33)	

¹, features of the retrospectively assembled cohort of 16 patients, whose genetic features were analysed. ², features of the patients of the retrospective cohort and cBioPortal database who harbored a *HER2* TMD mutation and had overall survival (OS) data. ³, features of the patients from cBioPortal database who harbored a *HER2* non-TMD mutation and had overall survival (OS) data. TMD, transmembrane domain; OS, overall survival; SD, standard deviation; LUAD, lung adenocarcinoma; LUSC, lung squamous carcinoma; KD, kinase domain.

harboring *HER2* V659E were diagnosed at an advanced stage (stage III–IV, n=13/16, 81.25%). Among the 16 concomitant genetic alterations (Figure 1C), *TP53* was the most frequently mutated gene (n=4, 25.0%) followed by *HER2* amplification

(n=2, 12.5%), *NSD1* (n=2, 12.5%) and *RARA* (n=2, 12.5%). The sequencing results of patient 02 and 10 suggested that *HER2* V659E could coexist with the driver mutations *EGFR* (p.T783I) and *BRAF* (p.I659M), though not at their

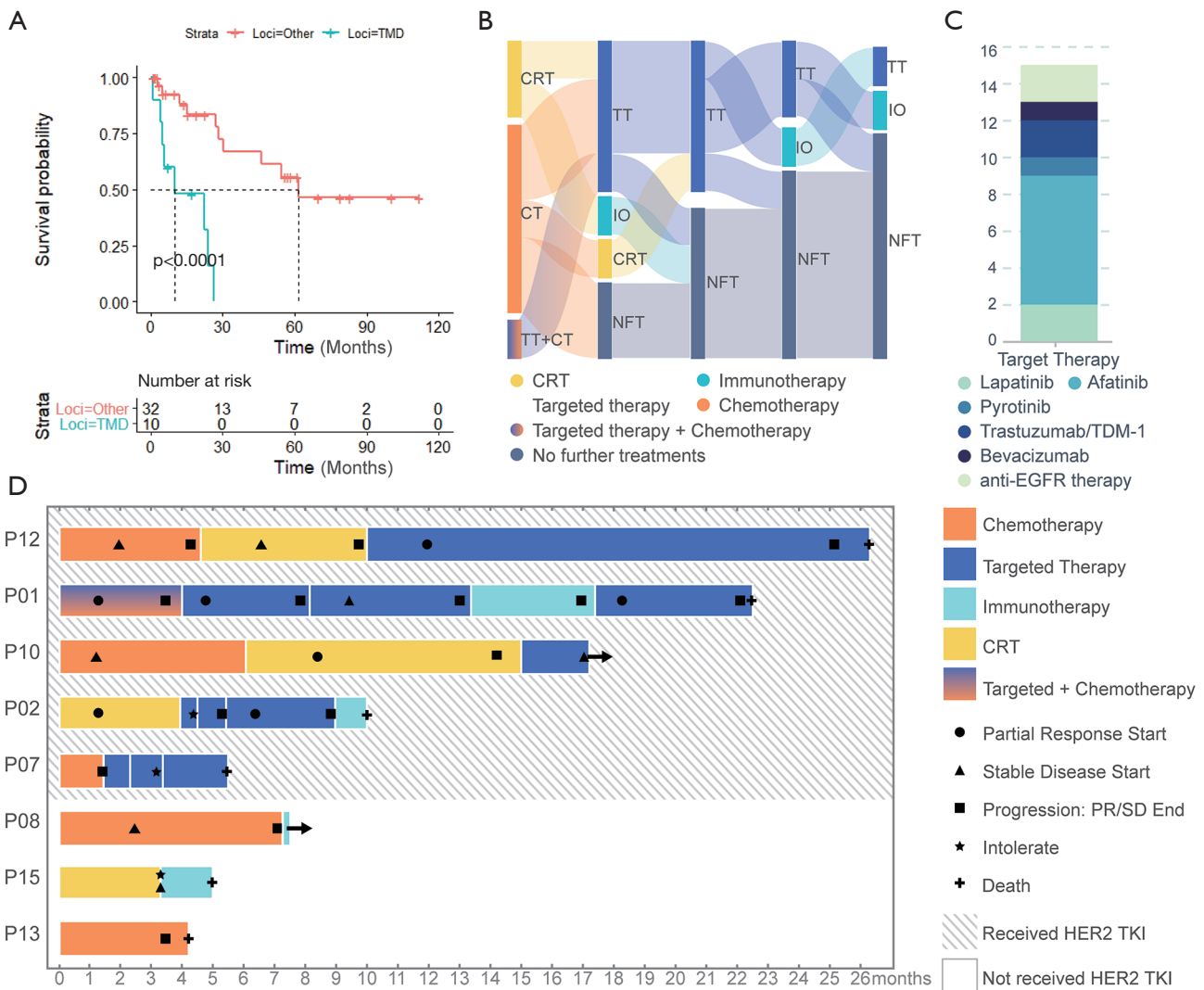


Figure 2 Overall survival and real-world treatment profile. (A) Overall survival in patients from *HER2* TMD and non-TMD mutation cohorts. Log-rank test: $P < 0.0001$. (B) Sankey diagram of treatment regimen of patients with *HER2* TMD mutations. (C) Targeted therapy used in the treatment regimen of patients with *HER2* TMD mutations. (D) Swimmers plot of time on treatment demonstrating PFS to each line of therapy and overall survival. Each bar represents one subject in the study. TMD, transmembrane domain; CRT, chemoradiotherapy; CT, chemotherapy; TT, targeted therapy; IO, immunotherapy; NFT, no further treatments; TKI, tyrosine kinase inhibitor.

hotspots. The eight patients from the in-house cohort with OS information together with two patients from the external cohort harboring *HER2* TMD mutations and with available OS were assembled into a cohort for further survival analysis. Thirty-six patients from the external cohort harboring *HER2* non-TMD mutation were also analyzed as a comparative cohort. These two cohorts were significantly different in the stage of diagnosis ($P < 0.001$) but were comparable from other perspectives.

Real-world evidence of treatments for *HER2* V659E patients and responses

Follow-up treatment information was available for eight *HER2* V659E-mutant patients from the in-house cohort, and OS data were available for all 46 patients harboring *HER2* mutations from both in-house and external cohorts. The Kaplan-Meier curves of patients from *HER2* TMD mutation and non-TMD mutation cohorts are shown in *Figure 2A*. The

Table 2 Multivariate survival analysis using Cox regression model in patients with *HER2* TMD or non-TMD mutation

Variables	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value
Age	0.97 (0.93–1)	0.24	–	–
Gender (male vs. female)	1 (0.4–2.5)	0.99	–	–
Pathology (LUSC vs. LUAD)	1.8 (0.68–4.9)	0.23	–	–
Smoker (no vs. yes)	1.2 (0.44–3.2)	0.73	–	–
Stage	1.9 (1.3–3)	0.0021	1.2 (0.61–2.5)	0.47
<i>HER2</i> (TMD vs. others)	9.3 (2.7–32)	0.00039	7.9 (1.03–61)	0.046

TMD, transmembrane domain; LUAD, lung adenocarcinoma; LUSC, lung squamous carcinoma; HR, hazard ratio.

median OS was 10.0 months (95% CI, 5.0–NA) for *HER2* TMD-mutant patients, and 61.6 months (95% CI, 30.1–NA) for patients harboring non-TMD *HER2* mutations. *Table 2* presents the hazard ratios (HR) associated with clinical and genetic characteristics. OS was significantly worse for *HER2* TMD-mutant patients than non-TMD-mutant patients (HR =7.9, 95% CI: 1.0–61.0, P=0.046). Stage was associated with survival but was not statistically significant in Cox multivariate survival analysis (P=0.47).

Most of the *HER2* TMD-mutant patients (7/8, 87.50%) received more than one line of treatment (*Figure 2B*) and all patients received chemotherapy or combination therapy including chemotherapy as their first-line treatment. Among treatment lines that used only chemotherapy or concurrent chemoradiotherapy, only two lines of treatment in two patients achieved partial response (ORR 22.2%, 2/9). Five patients received targeted therapy as at least one line of therapy and four of these achieved longer OS than the others. However, the choice of targeted therapy was not quite standard. Two patients received EGFR-TKI gefitinib, which was not guideline-recommended, and did not respond (*Figure 2C*). One patient received anti-angiogenesis agent bevacizumab, albeit for only one month before disease progression (*Figure 2C*). The overall response rate (ORR) of targeted therapy in assessable treatment lines was 57.1% (4/7). The longest progression-free survival (PFS) was 16 months, which was achieved by targeted therapy (afatinib) in P12 (*Figure 2D*). However, afatinib was also the most intolerable treatment, and two patients stopped using it because of severe oral ulcers, diarrhea, vomiting, or rash. Four patients received immunotherapy alone as a line of therapy and none of these achieved partial relief during or

after the treatment (ORR 0.0%).

A case report of a patient who is responding to pyrotinib as a fifth-line therapy

Patient 01, a 50-year-old Chinese woman with no smoking history, was diagnosed with stage IVa (pT2bN3M1a) LUAD with a rare *HER2* V659E mutation (*Figure 3*). PET-CT revealed a 4.5 cm × 5.3 cm × 3.4 cm mass in the right lower lobe, small, scattered nodules in the left lung, lymph node metastases, and a large amount of malignant pleural effusion. She was treated with lapatinib (1,250 mg, once daily) and capecitabine (2,000 mg, divided into twice daily) as first-line therapy, achieving a partial response (PR) with a PFS of 3 months, after which she presented with fast progression in her primary tumor and chest wall metastasis. Subsequently, afatinib (40 mg daily) was administered as second-line therapy and achieved PR with a PFS of 5 months. At progression, her bone and subcutaneous metastases developed, and subcutaneous tumor tissue samples were sent for NGS testing revealing novel amplifications in both *HER2* and *CCNE1*. Her treatment was then switched to TDM-1 (150 mg daily) and her pain was quickly relieved, but she rapidly progressed to atelectasis and hemoptysis and was subsequently administered with pembrolizumab (90 mg every 3 weeks). While her hemoptysis and pain were significantly alleviated in a week, despite a low tumor mutation burden (TMB), CT showed inflammation and no improvement in the primary nor the metastasized nodules in the lungs and new lesions were seen in the liver, adrenal gland, and abdominal wall. She then received a novel dual EGFR/*HER2* inhibitor, pyrotinib, achieving fast reduction of ascites and pain relief and her primary tumor

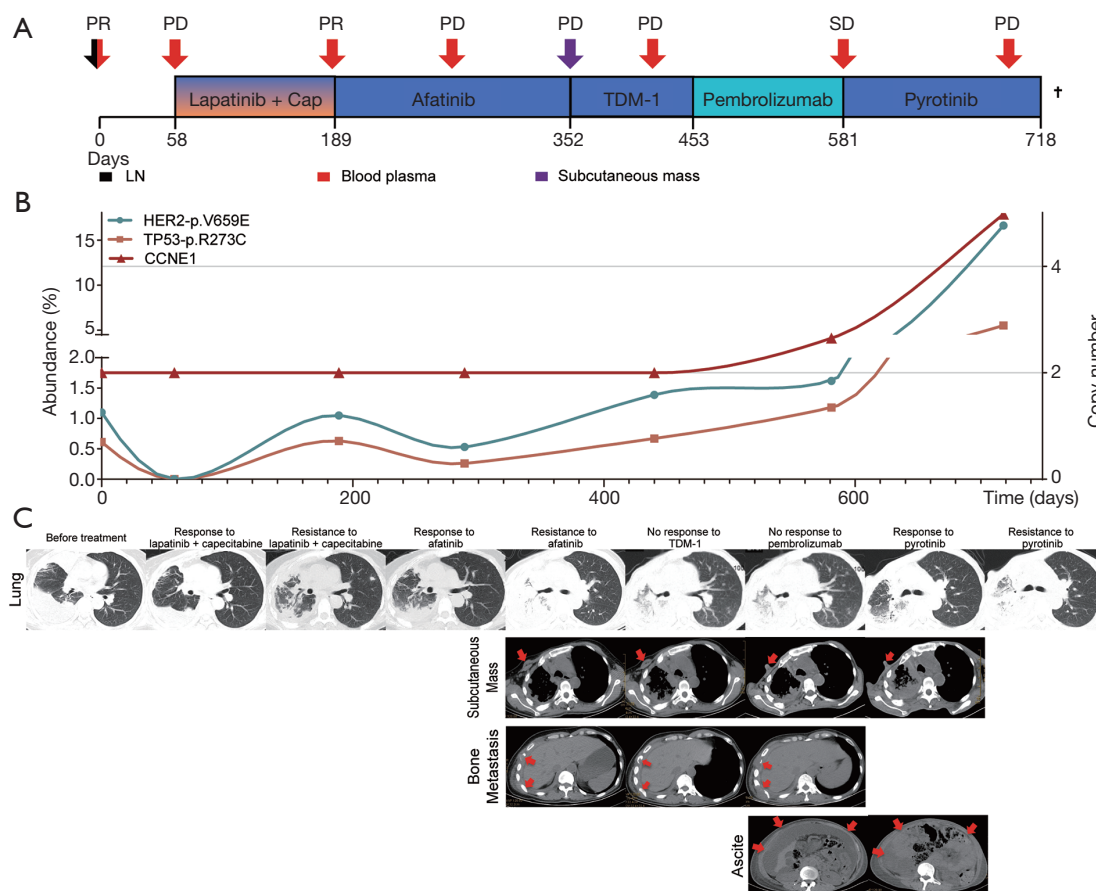


Figure 3 Genomic profiling and treatment regimen of Patient 01 and Docking simulation of *HER2* tyrosine kinase inhibitor. (A) Treatment regimen of patient 01, the duration of each treatment, and the abundance of mutation detected in lymph nodes (LN), pleural effusion, subcutaneous mass, and ascites by next generation sequencing under the various treatments. (B) The dynamic change in circulating tumor DNA (ctDNA) abundance demonstrating the evolution of the patient's tumor. (C) Computed tomography images of the patient's primary lung cancer, metastatic lung, subcutaneous mass, bone disease, and ascites before and after treatments. Subcutaneous mass and bone metastasis developed during progression disease when the patient was taking afatinib and cancerous ascites developed during disease progression when the patient was taking pembrolizumab (lapatinib, 1,250 mg oral daily; capecitabine, 2,000 mg oral divided into twice daily; afatinib, 40 mg oral daily; TDM-1, 150 mg oral daily; pembrolizumab, 100 mg i.v. every 3 weeks; pyrotinib, 400 mg oral daily). The red arrows show subcutaneous metastasis, liver metastasis, and ascites. (A, B, C are accordant in time on the x axis). TMD, transmembrane domain.

and metastatic tumors in the lungs and adrenal gland shrank. Metastatic lesions of the chest wall, bones, subcutaneous tissue, liver, and abdominal wall were also stable. After 3 months of PR, a fast progression in the lung was observed accompanied by the development of ascites and a significant increase in circulating tumor DNA (ctDNA) mutation abundance. The patient passed away shortly after.

Structure and energy analysis of lapatinib, afatinib, and pyrotinib in *HER2* kinase domain binding

Lapatinib is a non-covalent inhibitor that interacts with

HER2 dynamically as Eq. [1], and afatinib and pyrotinib are covalent inhibitors whose *HER2*-interaction follows Eq. [2] (E = target, I = inhibitor, $[EI]$ = non-covalent binding state of the target and the inhibitor, EI^* = covalently bonded state of the target and the inhibitor). Thus, afatinib and pyrotinib are more favorable for blocking *HER2* KD from being continuously phosphorylated than lapatinib. Between the two covalent inhibitors, pyrotinib ($\Delta G_{\text{Pyrotinib}} = -65.92$ kcal/mol) shows stronger non-covalent binding ability than afatinib ($\Delta G_{\text{Afatinib}} = -59.71$ kcal/mol). ΔG calculations of the three TKIs are shown in Table S5 and docking simulation is shown in Figure 4A,B,C.

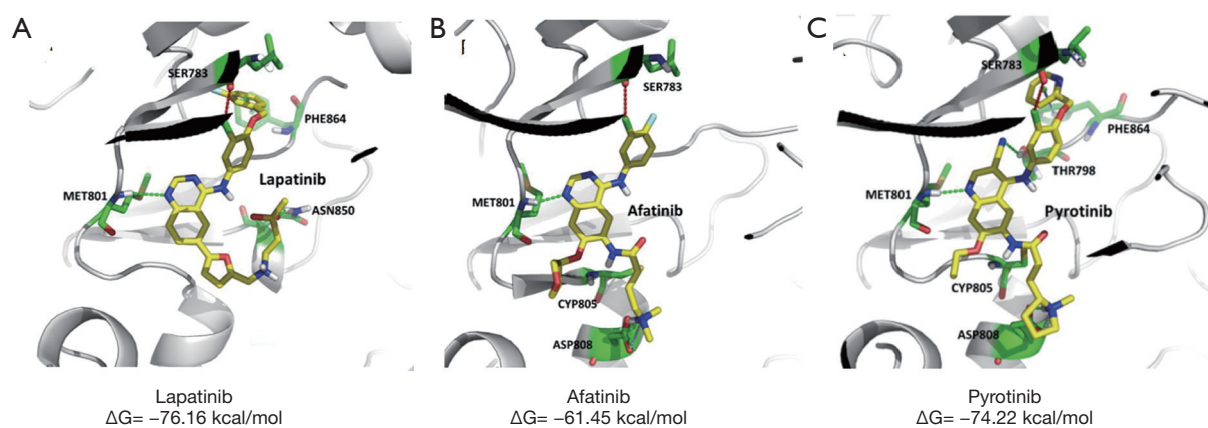


Figure 4 Docking simulation of lapatinib, afatinib, and pyrotinib in the *HER2* kinase domain. The ATP-binding pocket of the *HER2* kinase domain in the modeled *HER2*-lapatinib (A)/afatinib (B)/pyrotinib (C) complex structure is depicted. Lapatinib, afatinib, and pyrotinib are shown as a stick and the structure of the *HER2* kinase domain is shown in tertiary structure. The binding free energy of lapatinib, afatinib, and pyrotinib is shown in the figure.



Discussion

In this study, the incidence of *HER2* TMD mutation was 0.18% (14/7,812) in the Chinese LUAD population. These TMD mutations were more frequent in NSCLC than other solid tumors, and with a higher prevalence in LUAD than lung squamous cell carcinoma (LUSC) (42,56). V659E was the most recurrent mutation compared to that found in colorectal cancer, which was I655V (57). The prevalence of V659E was 0.14% (11/7,812), higher than the mutation rate in the external cohort derived from the TCGA database and ten other studies through cBioPortal (0.10%, 3/2,966) (27-37). The prevalence was also higher than 0.009%, which was reported by Ou *et al.* in 2017 (15). This variation might be explained by differences in the size or race of the investigated cohort. In addition to our current knowledge of all *HER2* KD mutations in published studies that occurred in LUAD (8), all TMD mutations identified in our study also were detected in LUAD and all the *HER2* V659E mutations in our cohort resulted from a dinucleotide

missense mutation. It was reported that *HER2* mutation occurred more frequently in younger women and non-smokers (6,8,11,58-60), and rarely co-existed with other lung cancer driver mutations (61). However, we observed no significant difference in gender or smoking history. We also found two patients harboring both *HER2* mutation and *EGFR* or *BRAF* mutations, respectively. This accorded with a previous study that non-KD mutations did not exclude concurrent driver mutations as KD mutations did (8).

Previous studies had reported a shorter OS in *HER2*-mutant lung cancer, especially KD mutations (8,12). The present study found that patients harboring TMD mutations had even shorter OS compared to *HER2* non-TMD mutations with statistical significance (HR 7.9, 95% CI, 1.03-61, $P < 0.001$), including KD mutations. It was also observed that patients harboring TMD mutations were diagnosed at a more advanced stage (HR 1.2, 95% CI, 0.61-2.5, $P < 0.001$), but were not significantly associated with a poorer prognosis.

In the present real-world retrospective cohort, although all patients had the same somatic gene mutation and similar clinical characteristics, their treatment regimens were diverse. For targeted therapy in *HER2* exon 20 insertion, *in vitro* cell-line study demonstrated that the most common YVMA insertion is sensitive to only neratinib, poziotinib, and pyrotinib (24,25). In the clinical setting, targeted therapy is the National Comprehensive Cancer Network (NCCN) guideline-recommended treatment for *HER2* mutation (4). The NCCN recommendation has evolved over time with trastuzumab, lapatinib, and pertuzumab being

recommended in 2013; trastuzumab and afatinib in 2014; and TDM-1 in 2018. However, the works cited to support these recommendations did not include patients harboring *HER2* TMD mutation until Li *et al.* published the results of their basket trial of *HER2*-mutant lung cancers (9). In that study, two V659E patients were recruited in the 18-patient cohort and only one of them achieved PR, while the disease progressed in the other (ORR 50%). In the present cohort, most of the patients received targeted therapies (ORR 57.1%), among which afatinib achieved the longest PFS of 16 months. However, two patients showed intolerance to afatinib and needed an appropriate substitution. Among patients who received targeted therapy, two patients progressed after receiving gefitinib. This suggested *HER2* V659E mutation did not respond to gefitinib, as was demonstrated previously *in vitro* (62). Three patients received immunotherapy, none of whom responded. Although it was previously reported that the response rate to anti-PD-1 immunotherapy in *HER2*-altered NSCLC was 7–35% (63–65), and was similarly low in other driver mutations, a larger cohort is needed to accurately determine the response rate of immunotherapy in *HER2* TMD-mutant patients. Chemotherapy and concurrent chemoradiotherapy were used in all patients (ORR 22.2%), and the efficacy was seen to be better than immunotherapy, although worse than targeted therapy.

We suggest the use of targeted therapy to rechallenge targeted therapy resistance. Previous studies reported short PFS in *HER2* TMD-mutant patients who responded to targeted therapies. The median PFS was 5 months in a basket trial treating *HER2*-mutant NSCLC patients with TDM-1 (9) and PFS was reported to be in the range of 3–18 months (median 5 months) in case reports of patients with *HER2* TMD-mutant patients treated with TKI (15,26,42). As we reported, a stage IV patient with multiple distal metastases achieved an OS of 22.4 months and obtained PR when receiving with first-line lapatinib with capecitabine, second-line afatinib, and fifth-line pyrotinib treatment. Each line of treatment had a PFS longer than 3 months. This might be a solution to conquer the short PFS of targeted therapy. We postulated that pyrotinib can conquer resistance to afatinib, and afatinib to lapatinib because of increased TKI binding affinity. Molecular dynamics simulation and binding free energy analysis were conducted to investigate the binding mode and strength of lapatinib, afatinib, and pyrotinib for the *HER2* kinase domain to further explain the sequential conquer of drug resistance in the reported case. The irreversible binding of covalent inhibitors to the target reduces the competition of

the endogenous substrate ATP on the kinase, which in turn provides more sustained kinase inhibition than non-covalent inhibitors (66,67). Thus, we conclude that the affinity of lapatinib, afatinib, and pyrotinib increases sequentially.

The efficacy of pyrotinib for tumors harboring an activating *HER2* alteration is being studied in a phase I basket trial including NSCLC (68). Another study reported that LUADs harboring *HER2* KD mutations could respond to pyrotinib better than afatinib (69). The results of our study suggest pyrotinib is also effective for advanced stage *HER2* V659E-mutant LUAD. More refined stratification of patients based on the type of *HER2* activating alterations might be needed in further studies of pyrotinib.

A limitation of this study is that it was a descriptive study based on a real-world multicenter retrospective cohort. The efficacy of targeted therapy, chemotherapy, and immunotherapy should not be directly compared. This limitation reflects the real-world challenge in assembling cohorts of *HER2* TMD-mutant patients because of the low prevalence. While basket trials might serve as a solution, the intra-cohort heterogeneity of prognosis might reduce its reliability in specific mutation subgroups. We suggest finer stratification and transparent data sharing policy in further studies to facilitate future meta-analysis of individual participant data, which is considered as the most accessible high-level evidence for low prevalence mutations.

Conclusions

The present study was the first to report the comprehensive profiles and real-world evidence of treatment responses in *HER2* TMD-mutant lung cancer patients and to report a case that rechallenged TKI resistance with stronger-affinity TKI and responded to fifth-line pyrotinib.

With the wide application of NGS in tumor evaluation and the inclusion of *HER2* in most NGS panels, more patients with *HER2* TMD mutations have been uncovered. As we have illustrated, patients harboring *HER2* TMD mutations have poorer survival than patients with other *HER2* mutations, highlighting the need for finer stratification of *HER2* mutations in future clinical studies.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in studies involving human participants were in accordance with the Helsinki Declaration (as revised in 2013). The study was conducted under the approval of the Ethics Committee of Peking Union Medical College Hospital (ZS-1329). Written informed consent was obtained from each patient.

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